Control of arthropods and the animal/plant pathogens they vector by transgenesis and paratransgenesis – status and future directions

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Transgenic approaches to arthropod control

- germ-line transformation of host arthropod genome

- types of control
  - improve biological control of pest species
  - make disease vectors refractory to pathogens
  - improve health and reproduction in beneficial insects

- methodology
  - transposon-mediated transformation
  - mass-rearing and field release sterile or autocidal transgenics for biocontrol
  - field release transgenics to replace disease vector population

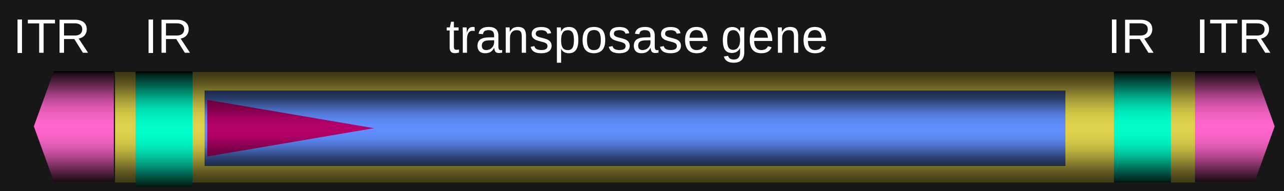
Paratransgenic approaches to arthropod animal/plant pathogen control

- transformation of bacterial or viral symbiont of arthropod host

- types of control
  - express antibodies, lytic peptides, RNAi, etc
  - directly target pathogen for death
    - Plasmodium, Trypanosome, protozoa, bacteria, virus
  - target arthropod to inhibit vectorial capacity

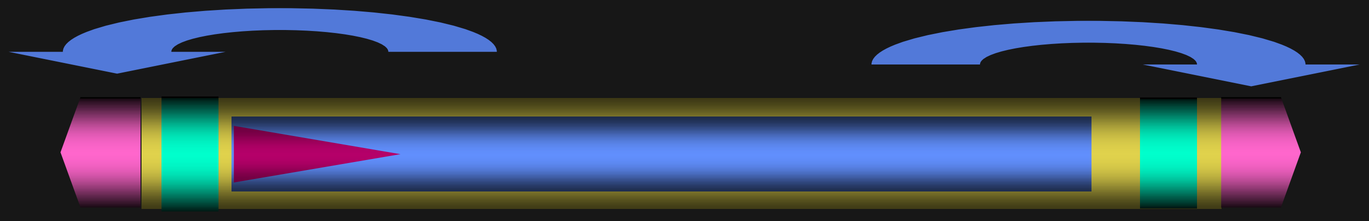
- methodology
  - transform symbiont
  - infect host (feeding/inoculation)
  - continued infection by trans-ovarial, venereal, or coprophagy

Transposon vectors for germ-line transformation



- Class II transposable elements
- DNA-mediated cut and paste transpositions
- 1.3 to 3 kb elements
- 10-30 bp short inverted terminal repeats (ITR)
  - sometimes sub-terminal inverted repeats (IR)
  - generally 5' and 3' termini are not interchangeable
- internal transcriptional unit encodes transposase
  - acts at or near ITRs *in trans*

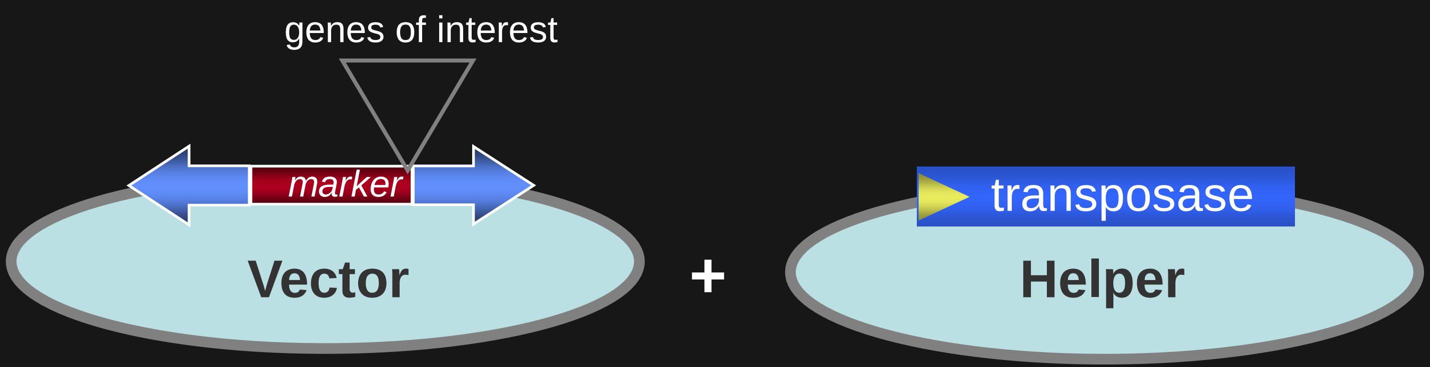
Transposon-based germline transformation



- transposase acts on transposon termini to ‘cut’ and ‘paste’

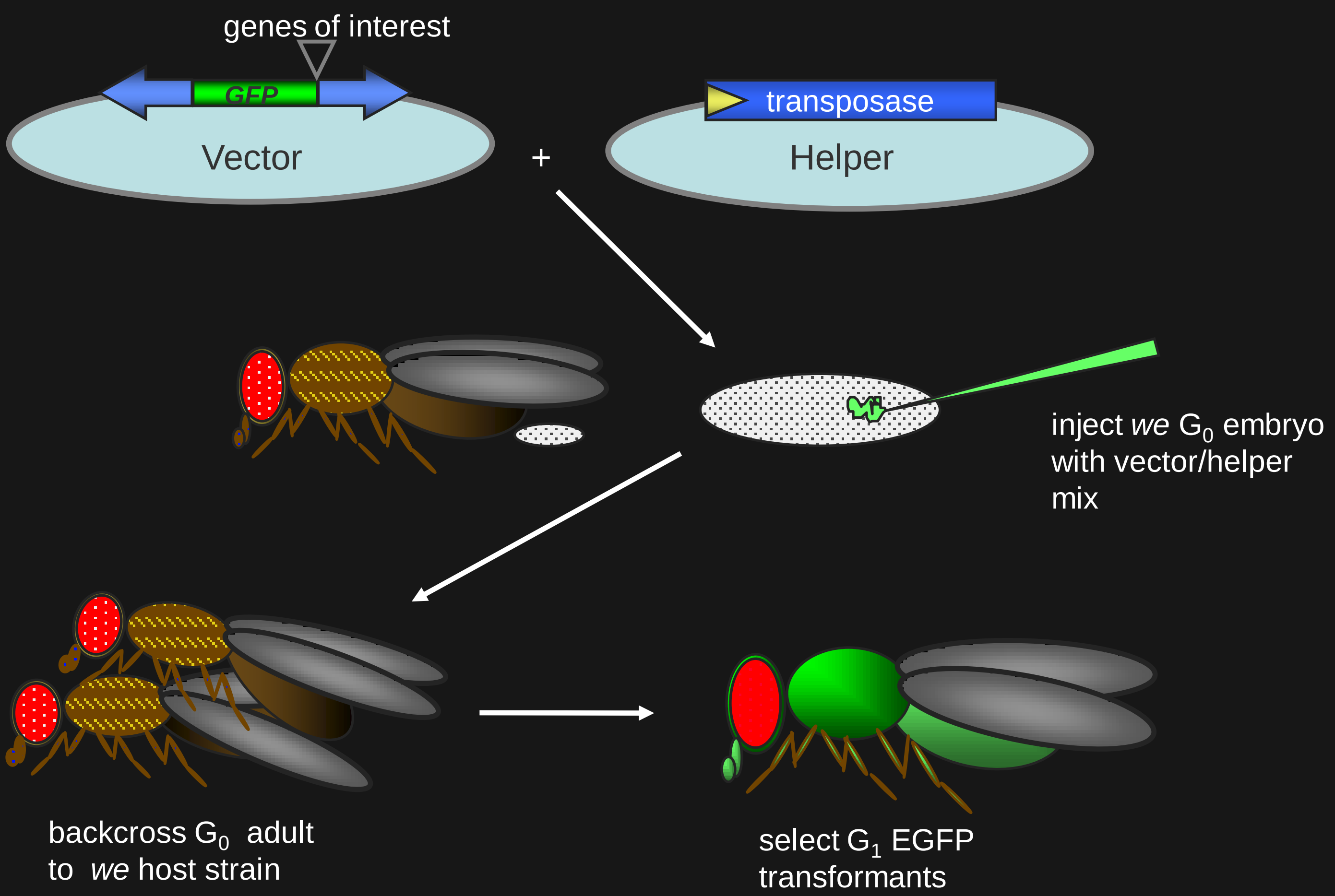
Binary transposon-based germline transformation

- first developed for *P* vectors (Rubin and Spradling 1984)



- vector has deleted or disrupted transposase
- helper has transposase without one or both termini - cannot integrate
- vector should be stable in absence of transposase

Transposon-based germline transformation



Transposon-based gene-transfer systems

Family	Transposon	Host species	Species transformed (incomplete)
P-element	*P*-element	*D. melanogaster*	*D. melanogaster, D. simulans*
hAT	*hobo*	*D. melanogaster*	*D. melanogaster, D. virilis*
	Hermes	*M. domestica*	*D. melanogaster, Ae. aegypti* *S. calcitrans, T. castaneum* *C. quinquefasciatus, C. capitata*
*mariner/Tc*1	***Mos1***	*D. mauritiana*	*Drosophila, Ae. Aegypti, M. domestica*, (chicken, zebrafish)
	Minos	*D. hydei*	*D. melanogaster, C. capitata* *An. stephensi*
TTAA	***piggyBac***	*T. ni*	*D. melanogaster, C. capitata, A. suspensa* *B. dorsalis, B. mori, P. gossypiella,* *An. albimanus, T. castaneum, Ae. aegypti,* *C. homonivorax, A. ludens* (mice, plasmodium)

Organisms transformed with *piggyBac*

- Invertebrates
  - >30 insects in 5 orders - flies, moths, beetles, hymenoptera
    - mosquitoes: *Anopheles, Aedes*
    - tephritid fruit flies: medfly, oriental, mexfly, olive, caribfly, etc
    - screwworm, sheep blowfly, red flour beetle, pink bollworm, silkmoth
  - *Plasmodium falciparum*
  - *Schistosoma mansoni*

- Vertebrates
  - mice, embryonic stem cells
  - pig and chicken cells
  - human fibroblasts, T-lymphocytes, embryonic stem cells

Transgenesis for Biological Control of Arthropod Pests

Biocontrol

- for agricultural and human health pests
- requires artificial mass-rearing and field release

Improvement of SIT

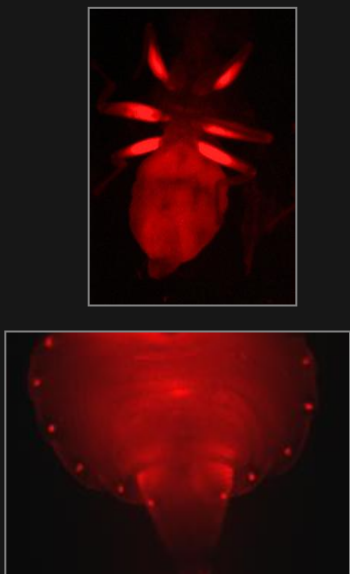
- genetic marking for field detection
  - sperm-marking to detect mated females
- genetic sexing for male-only strains
- male sterility

New autocidal strains

- strains for conditional lethality
  - non-sex-specific death of progeny in field
  - female-specific lethality in rearing
    - for male-only release

Fluorescent proteins for genetic marking

(*polyubiquitin*-regulated DsRed/EGFP)

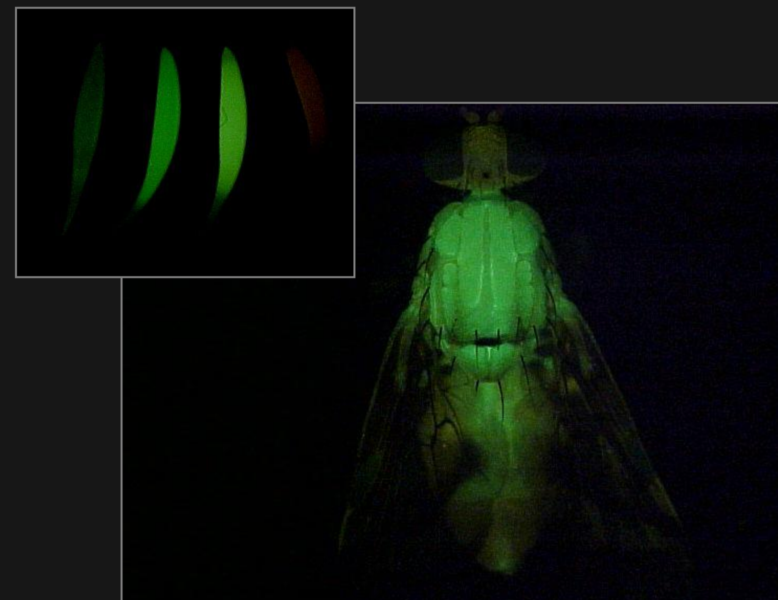


Ceratitis capitata
Mediterranean fruit fly

wild type transformed

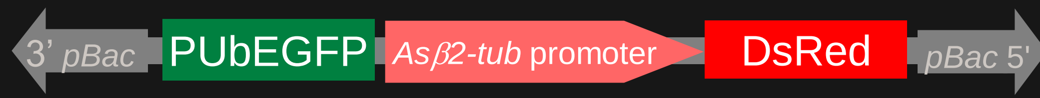
Bright field

DsRed epifluor.



Anastrepha ludens - Mexican fruit fly

piggyBac transformation vector for testis-specific fluorescent protein expression using *A. suspensa* promoter



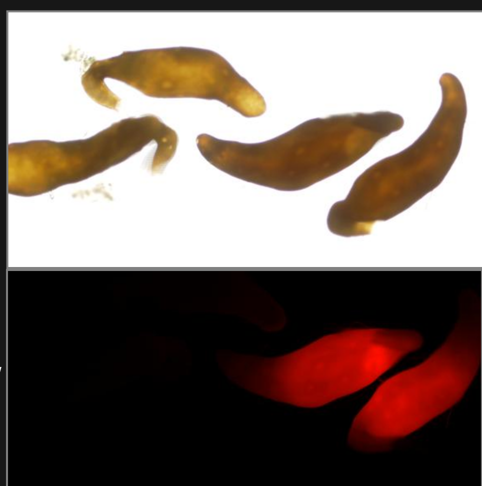
pB[PUbnlsEGFP/Asβ2tub:DsRed]

<u>fluorescent testes</u>

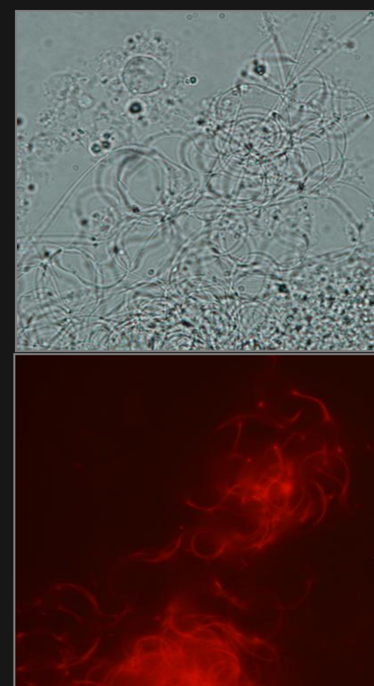
wild type transformed

Brightfield

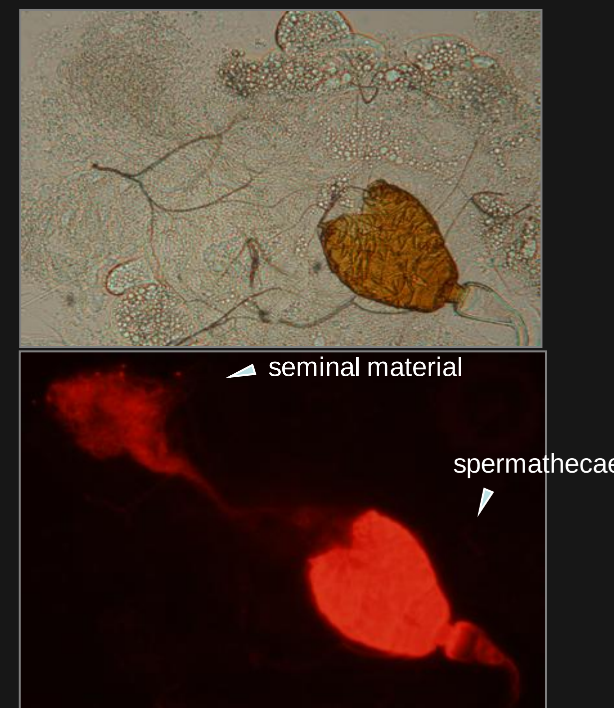
Texas Red/ DsRed



<u>fluorescent sperm</u>

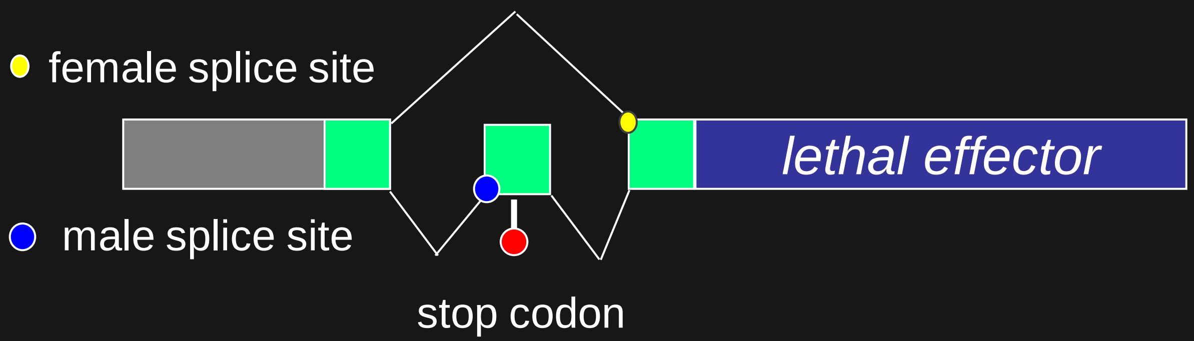


<u>sperm from female spermathecae</u>



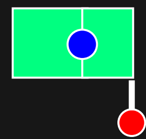
Female-specific lethality by *tra* alternative mRNA splicing

tra intron splice cassette



Males - stop codon not spliced - lethal effector truncated and non-functional

➢ Males survive!



Females - stop codon spliced out with intron - lethal effector produced

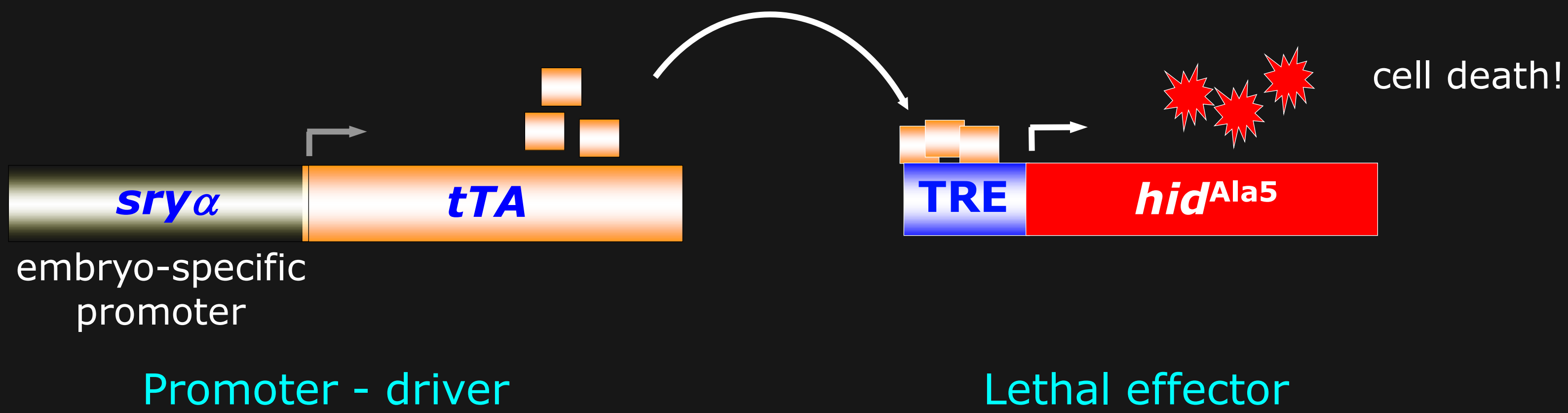
➢ Females die!

Conditional Lethality for Autocidal Biocontrol

- lethal genes can be used for organismal, tissue, or sex-specific death
- necessary to conditionally regulate lethal gene expression to maintain breeding strains
- Tetracycline suppression (tet-off) of gene expression
- Temperature-dependent regulation
  - *heat shock protein (hsp)* promoter
  - temperature-sensitive toxin genes
  - temperature-sensitive dominant lethal gene mutations

Tet-off conditional embryonic lethality

- transgene based, suppressible embryo-specific lethality system
- developed in Drosophila and implemented in medfly



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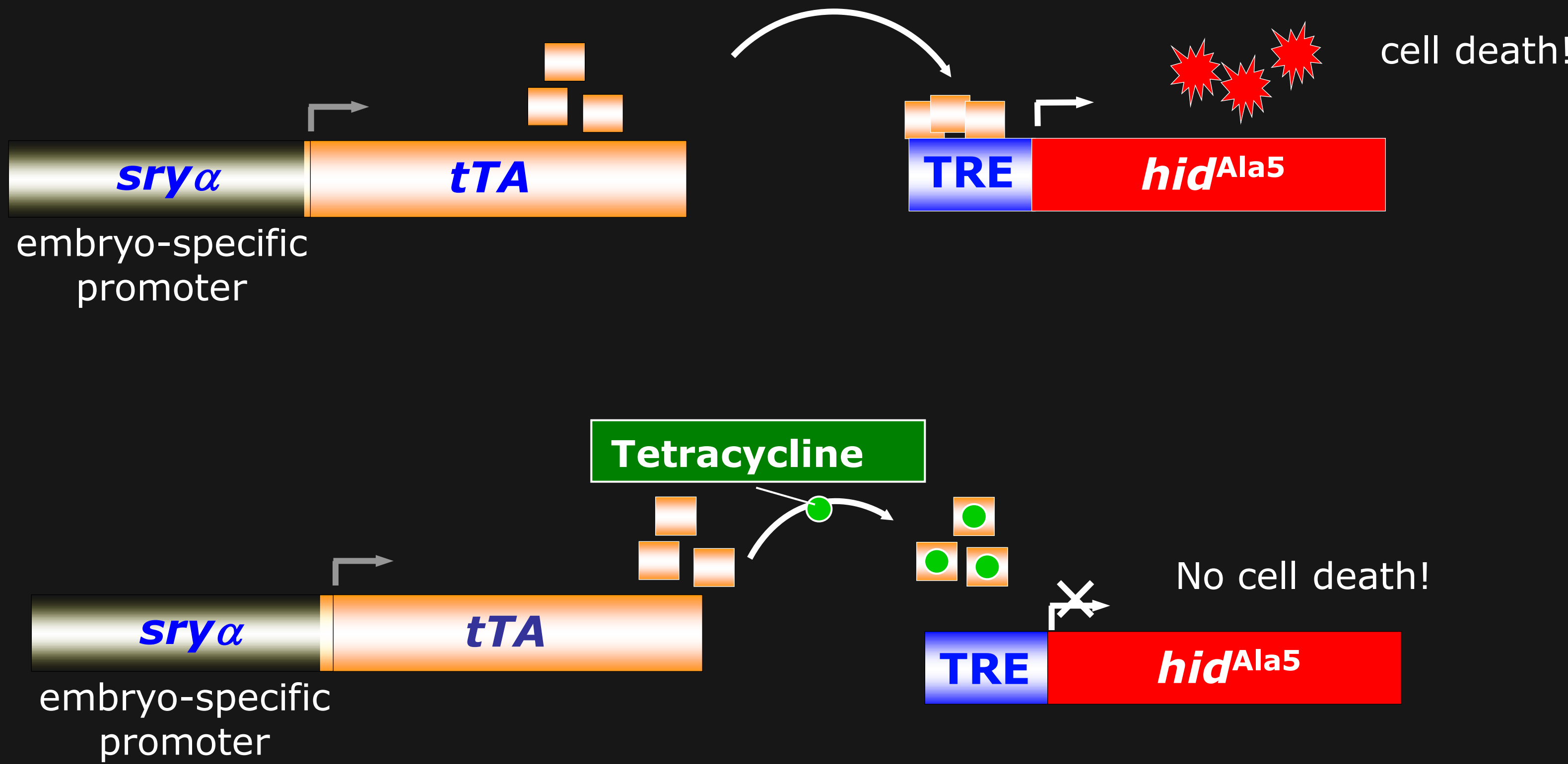

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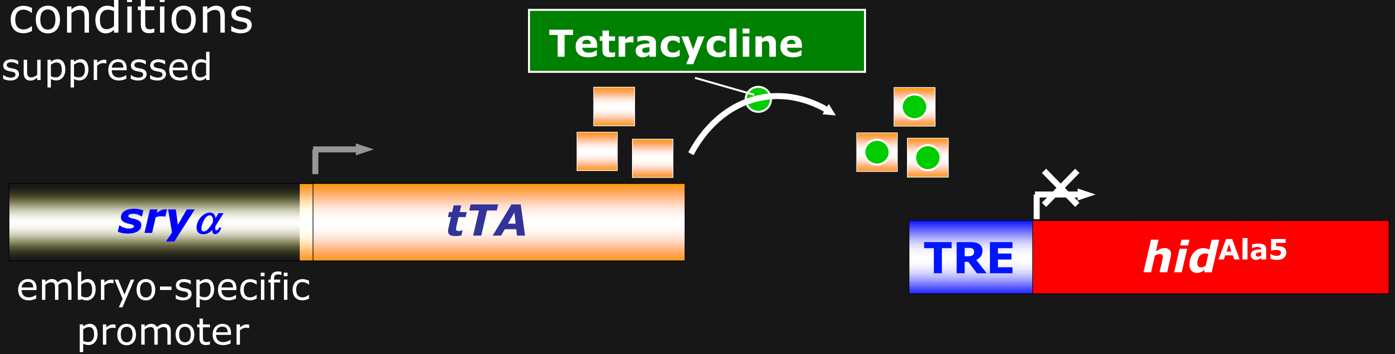
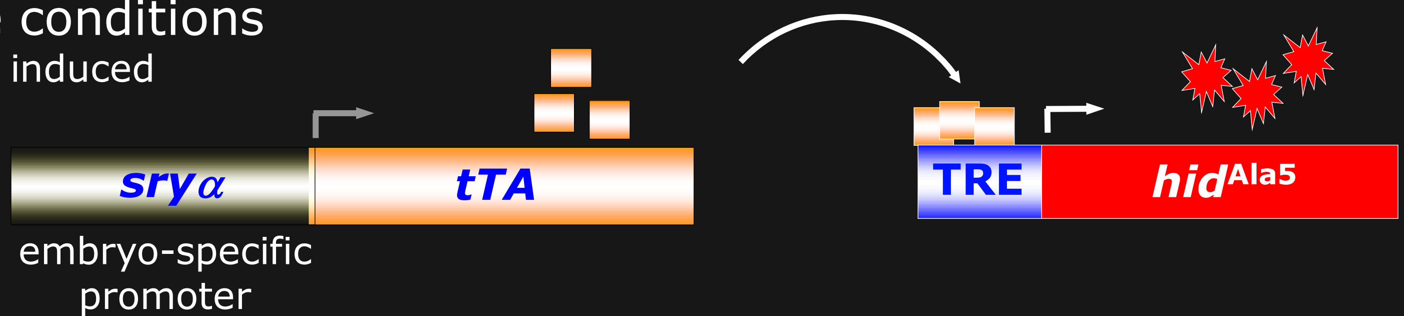
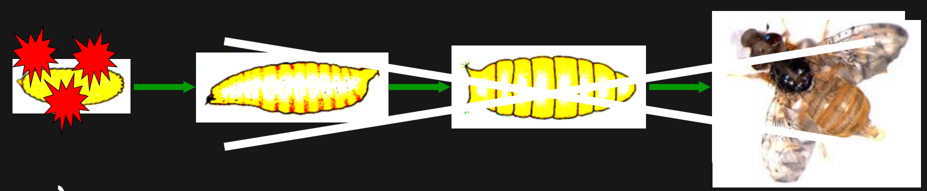
- developed in Drosophila and implemented in medfly

Rearing conditions
- lethality suppressed



Release conditions
- lethality induced





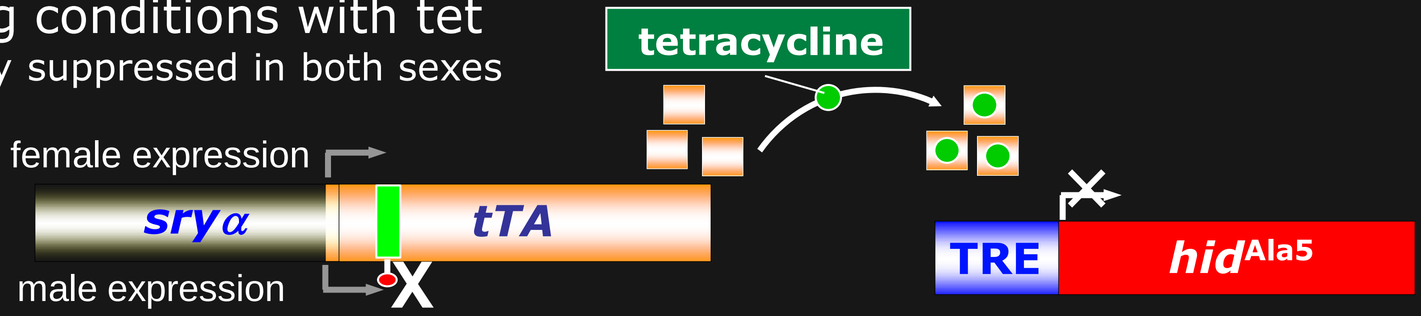
Horn & Wimmer, 2003, Nature Biotechnology
Schetelig et al., 2008, BMC Biotechnology

100% lethality in Drosophila and medfly

Tet-off female-specific conditional embryonic lethality

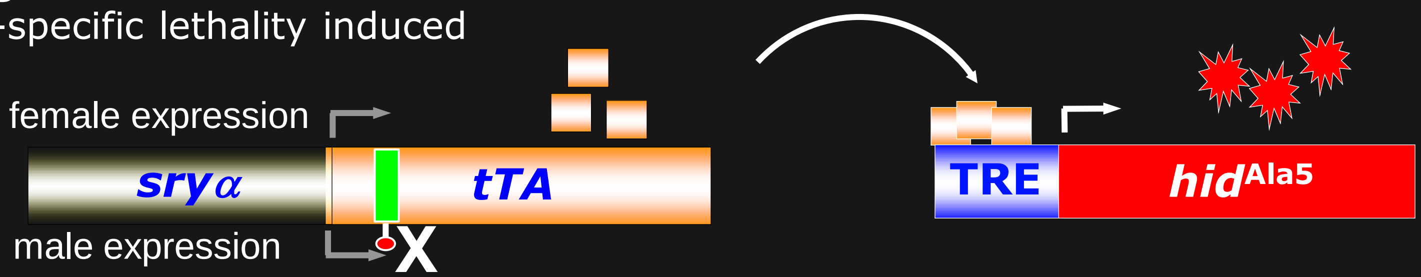
Rearing conditions with tet

- lethality suppressed in both sexes

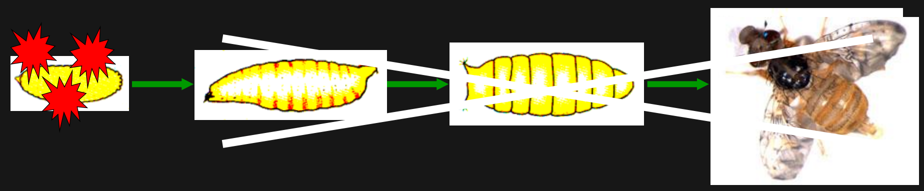


Rearing conditions without tet

- female-specific lethality induced

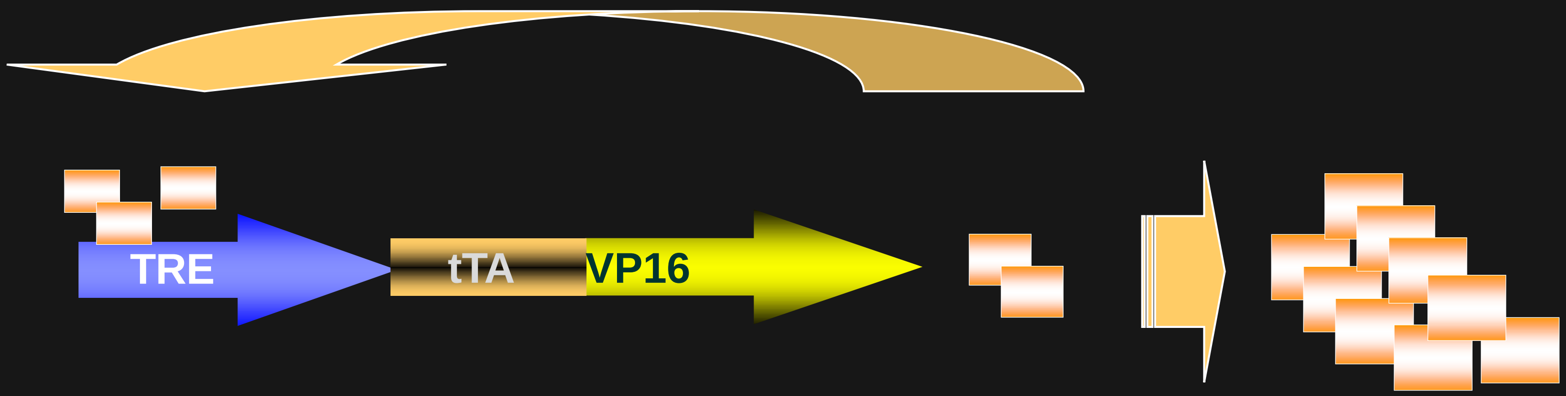


- only female embryos die without tet



Tet-off conditional regulation of larval/pupal lethality

RIDL - release of insects with dominant lethality (Oxitec Ltd.)
- medfly, mexfly, *Ae. aegypti*



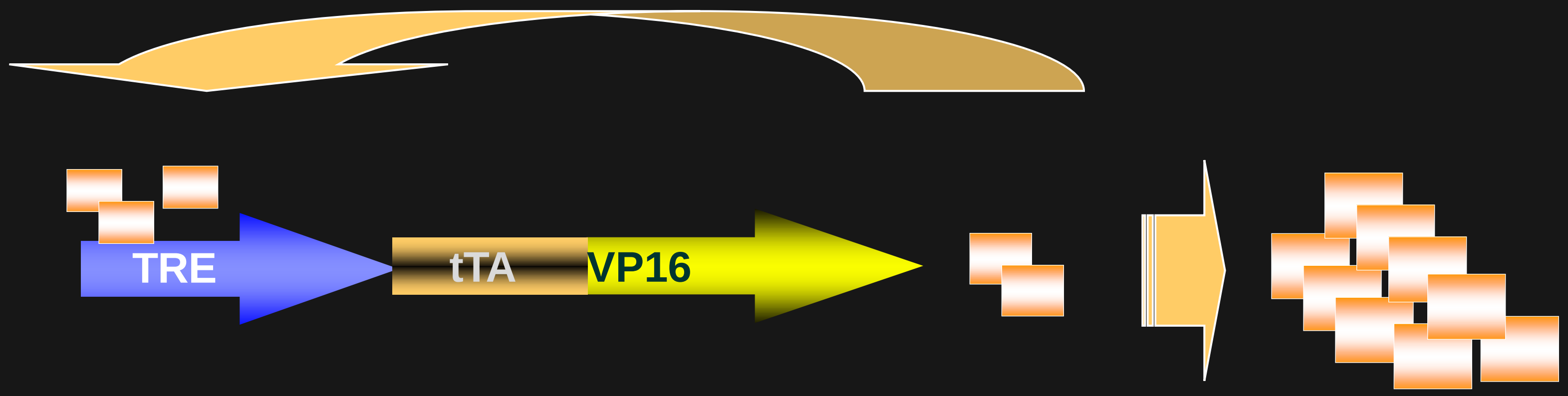
TRE promoter linked to tTA has tTA driving its own expression

Accumulation of tTA results in larval/pupal lethality

- effective in mosquitoes - adult vectors
- less effective for tephritids – larval feeding

Tet-off conditional regulation of larval/pupal lethality

RIDL - release of insects with dominant lethality



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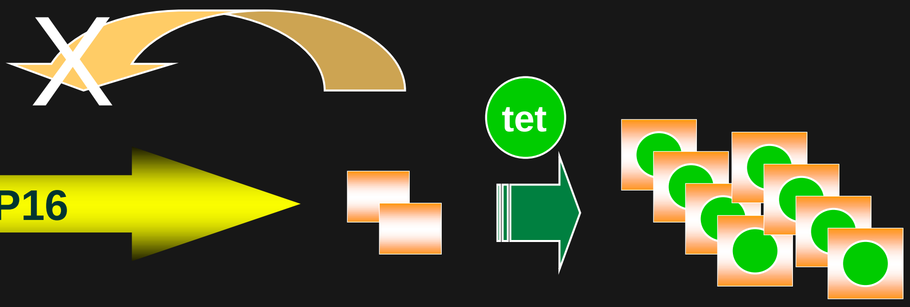


Tetracycline inhibits tTA binding to the TRE blocking tTA accumulation

Dominant Temperature Sensitive Lethality for Biocontrol

- introduce dominant mutation that causes death in larvae/pupae at 29-30^{o}C
- rear insects at 25^{o}C or below and release homozygous males
- all heterozygous offspring die at elevated ambient temperature
- system useful for tropical and subtropical pests
- existing mutations include DTS-5 (*Pros 26*^{1}) and DTS-7 (*Pros β2*^{1})
    - proteasome 20S subunit mutations cloned from Drosophila

Dominant Temperature Sensitive Lethality for Biocontrol

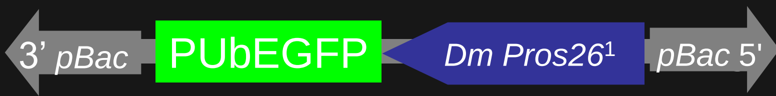
- introduce dominant mutation that causes death in larvae/pupae at 30ºC
- rear insects at 25ºC or below and release homozygous males

all heterozygous offspring die at elevated ambient temperature

- system useful for tropical and subtropical pests
- existing mutations include DTS-5 (*Pros 26*^{1}) and DTS-7 (*Pros β2*^{1})
    - proteasome 20S subunit mutations cloned from Drosophila

Dominant temperature sensitive lethality using *Drosophila* DTS-5 mutation

(John Belote, Syracuse University)

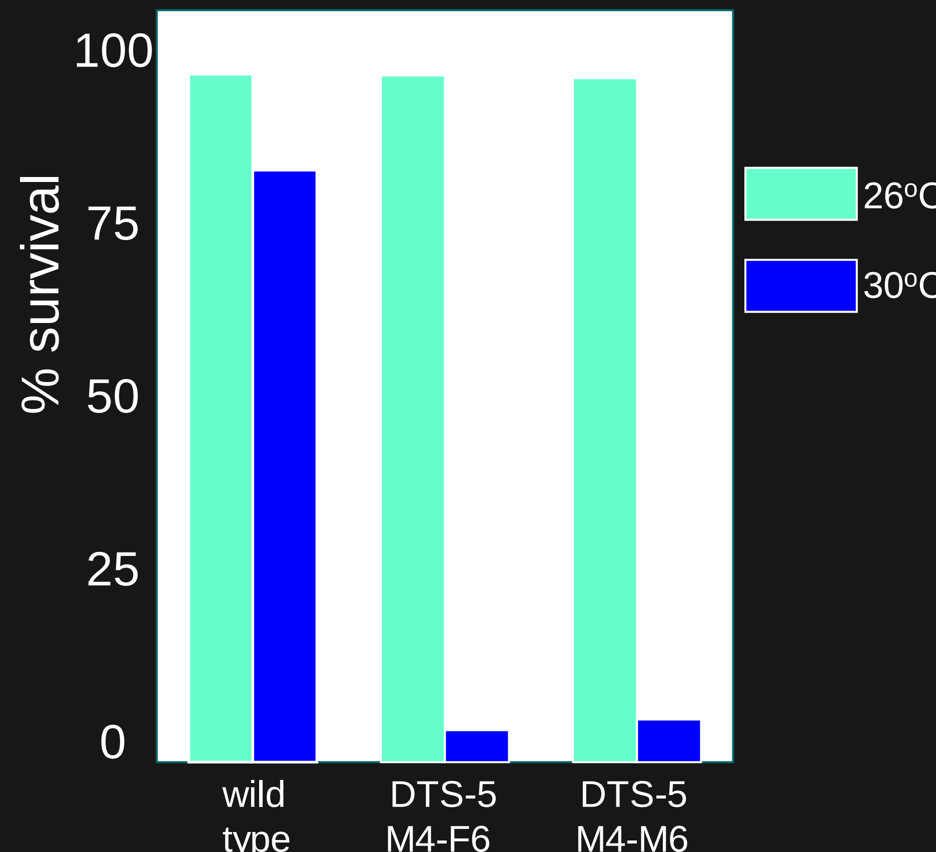


- Medfly transformed with *piggyBac*/EGFP/DTS-5 vector
- 90-95% lethality in homozygotes (2 doses) at 30°C
- need multiple integrations for heterozygous lethality

Caribfly larvae reared at 30°C

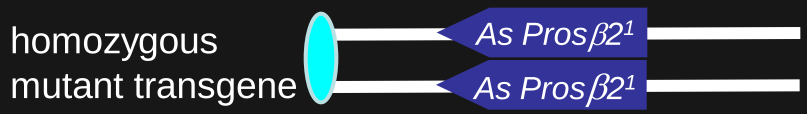
wild type

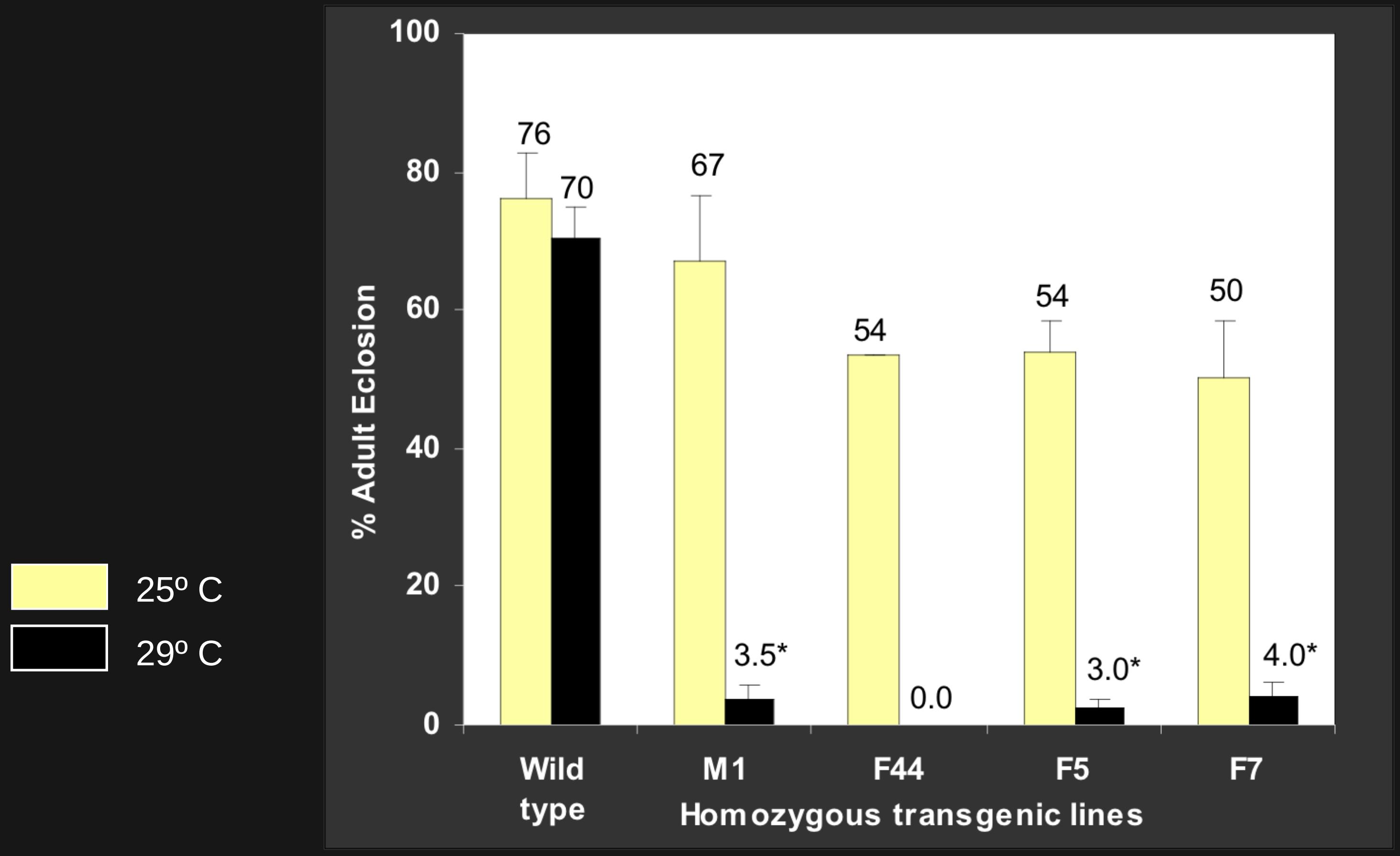
hspDTS-5 transformants



Pupal lethality in *A. suspensa* homozygous for the mutant *AsProsβ2*[1] transgene

(*n* = 994 to 1282 at 29º C)



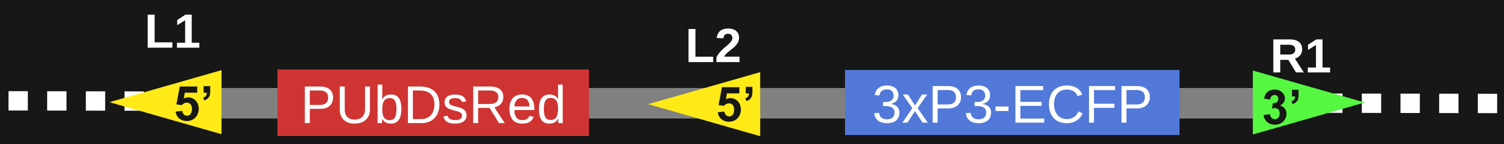


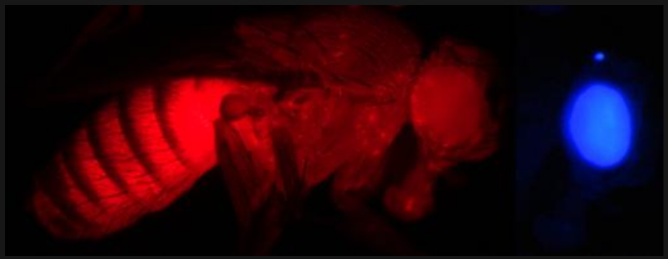
Major issues for development and release of transgenic insects

- transgene stability
  - strain integrity and ecological safety

- random integrations
  - mutations affecting strain fitness
  - position effects affecting transgene expression

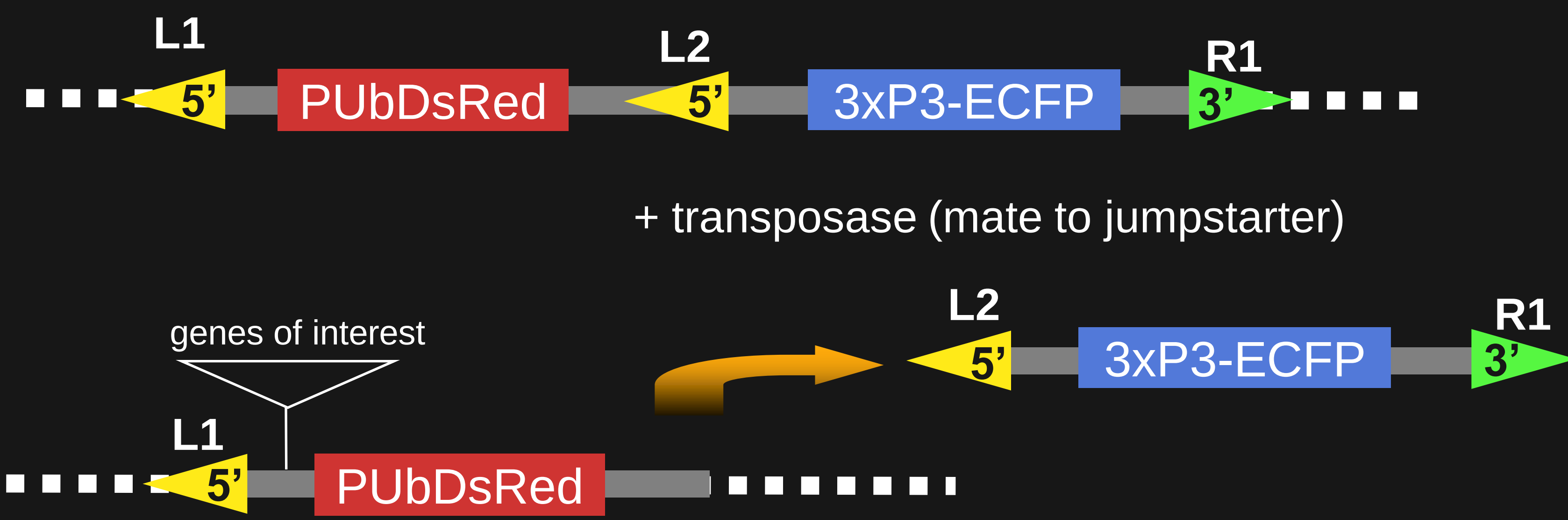
Vector stabilization by terminus deletion





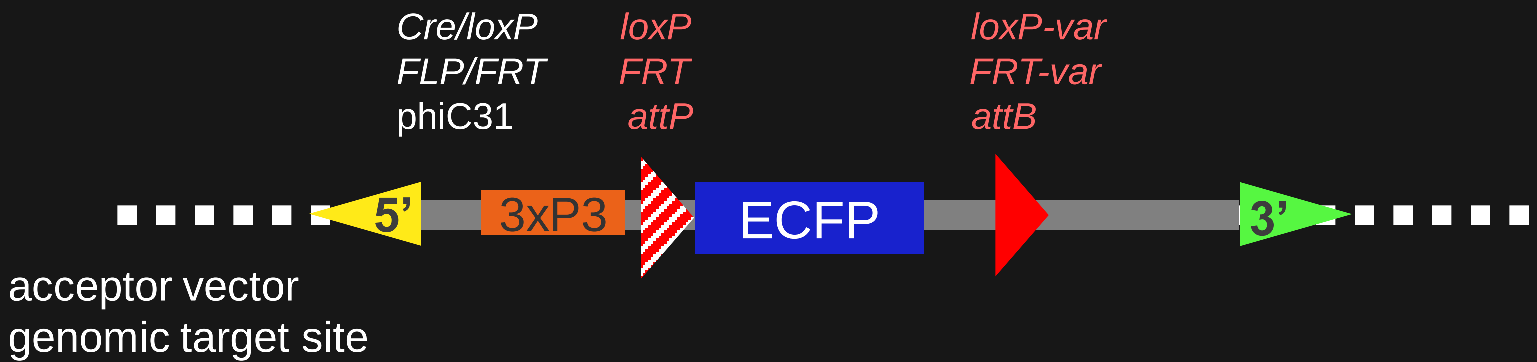
Vector stabilization by terminus deletion

- remobilize internal vector by jumpstarter mating or helper injection
- excision deletes 3’ terminus - loss of ECFP marker
- remaining DsRed marker/GOI + 5’ end is stable - cannot be remobilized!!



Vector targeting by recombinase-mediated cassette exchange (RMCE)

- use dual non-interacting heterospecific recombinase/integrase sites for double recombination



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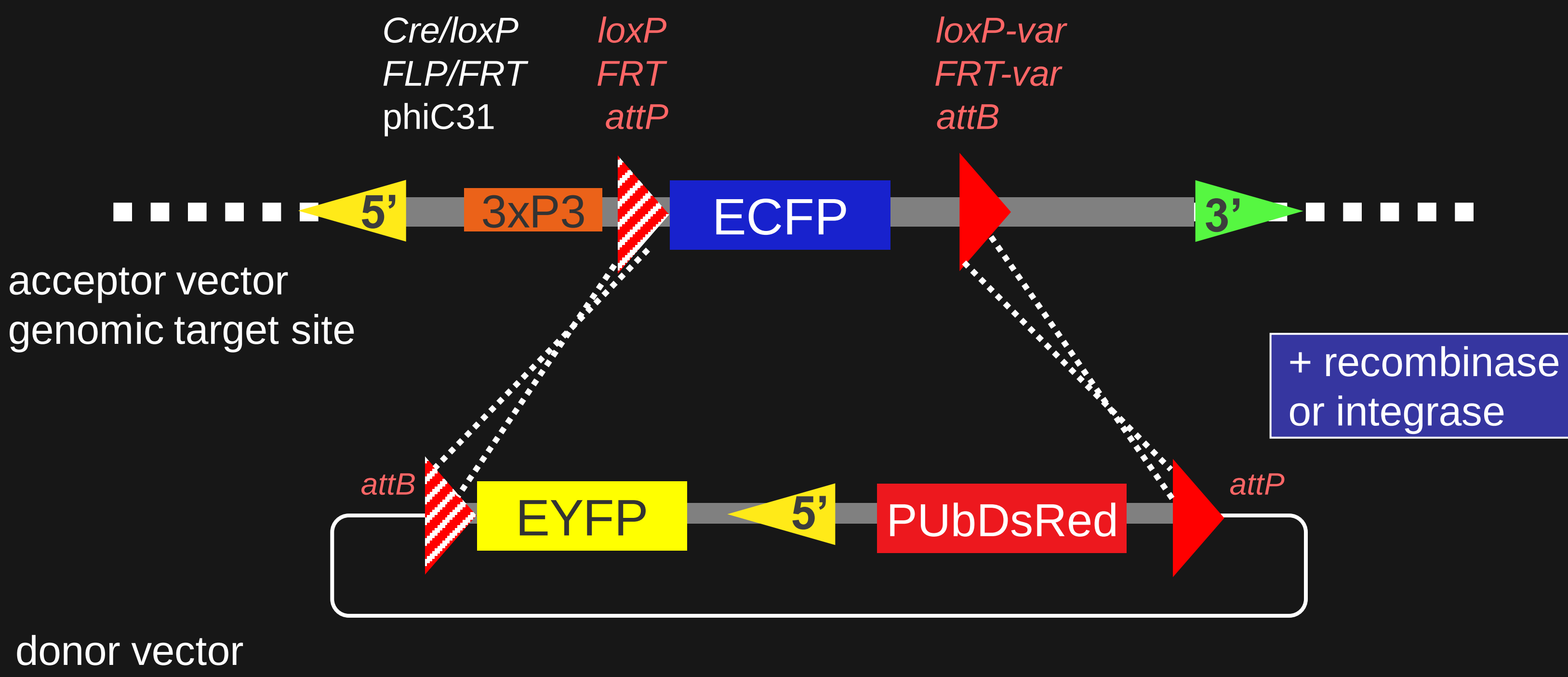

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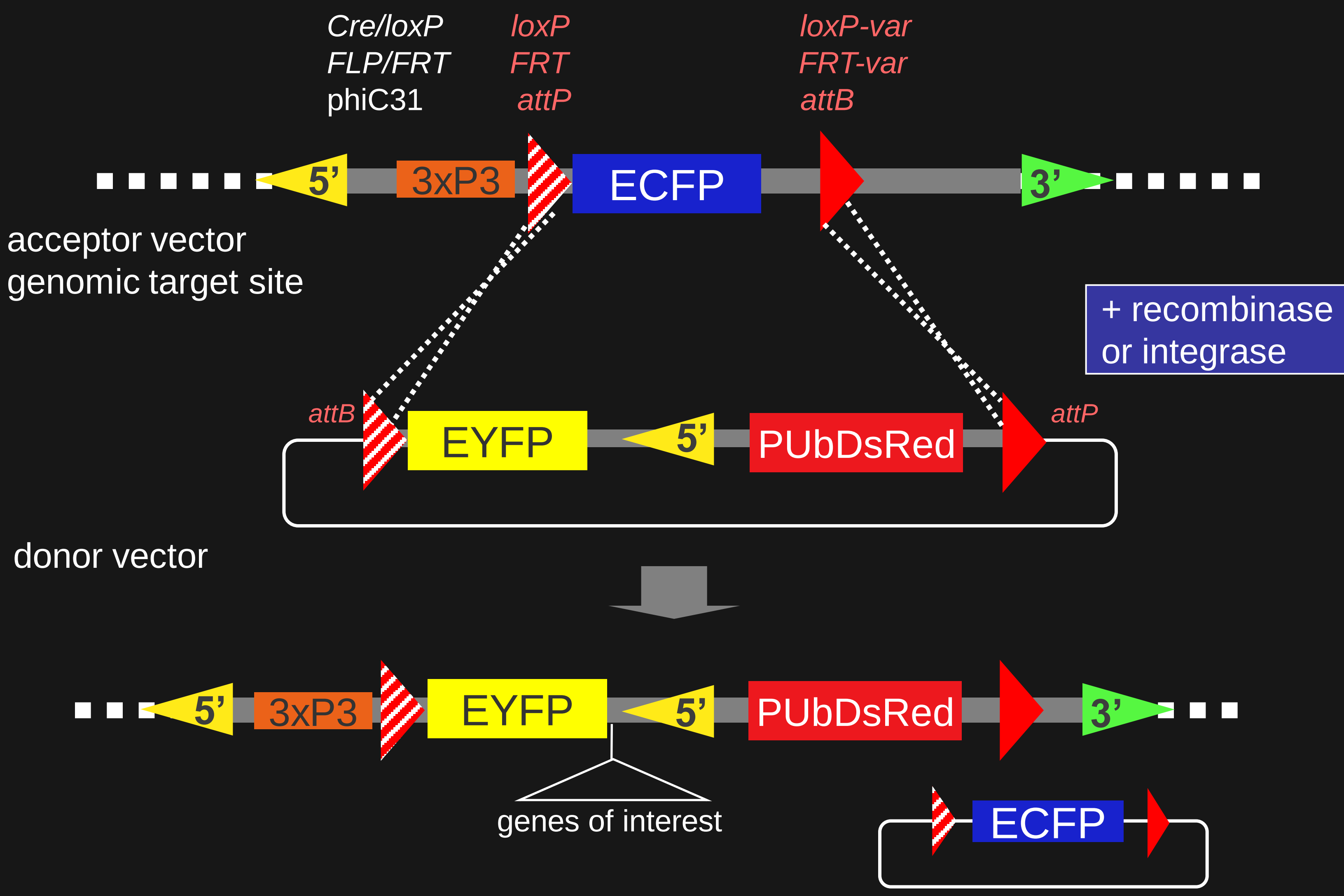


Transgenesis to control vectors of animal and plant disease

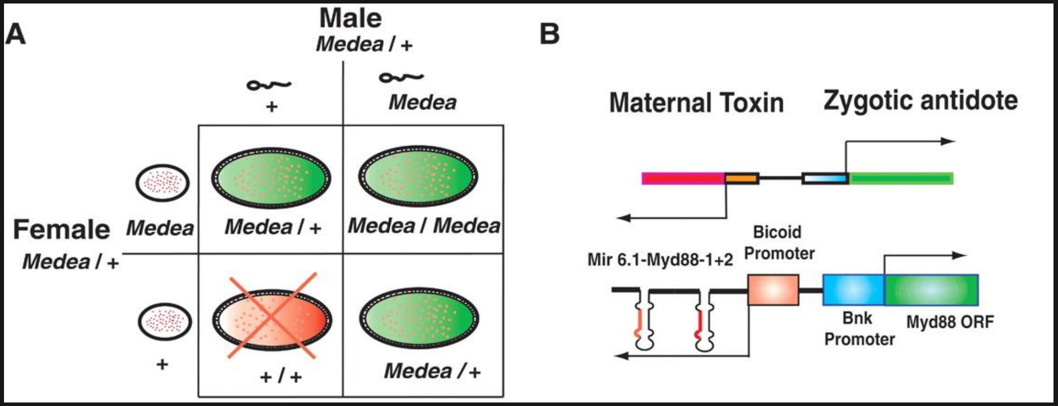
- strategies to control vector insects *not* amenable to mass-rearing and release
- transform insect vector of animal or plant disease to kill pathogen
  or interrupt its transmission
    - direct expression of interfering peptide to site of pathogen interaction
    - requires tissue-specific promoters
- eventual goal to replace existing vector population with 'innocuous' transgenics
    - requires some type of 'gene drive' system
    - *Medea* 'selfish gene' system is most promising

Transgenesis to target animal disease pathogens

Host species	Promoter	Transgene	Target	Pathogen
Ae. aegypti	*Ae vitellogenin*	*Defensin A*	fat body	*Micrococcus luteus* *Enterobacter cloace* *P. gallinaceum*
Ae. aegypti	*Ae vitellogenin*	*Cecropin A*	fat body	*Enterobacter cloace*
Ae. fluviatillis	*Ag peritrophin*	*mphospholipase-2*	midgut	*P. gallinaceum*
An. gambiae	*Ae carboxypeptidase*	*Cecropin A*	midgut	*P. berghei* (61% oocyst reduction)
An. stephensi	*Ag carboxypeptidase*	*phospholipase-2*	midgut	*P. berghei* (87% oocyst reduction)
An. stephensi	*Ag carboxypeptidase*	[SM1]4	midgut	*P. berghei*
An. stephensi	*Ag peritrophin*	*phospholipase-2*	midgut	*P. berghei*

Maternal effect *'Medea'* selfish-gene drive system

(Chen et al. 2007, Science *316*, 597)



- synthetic *'Medea* (*M*) element contains 'toxin' and 'antidote'
  - toxin is maternal microRNA to *Myd88* zygotic gene 5'UTR
  - antidote is zygotic *Myd88* ORF lacking the 5'UTR
- Heterozygous *M*/+ females give maternal toxin to all oocytes
  - only zygotes that are *M/M* or *M*/+ have antidote and survive
  - +/+ non-*Medea* zygotes without antidote die
- gene drive system has transgene linked to *Medea*

Status, challenges and future directions for use of transgenesis to control arthropods and their pathogens

Status

Biocontrol:

- transgenic strains for SIT/autocidal biocontrol are well-developed
    - large-scale and/or field testing in progress or being planned
- NAPPO framework in place to test and release transgenic insects
- new vectors for transgene stabilization and targeting available

Anti-pathogenic:

- proof of concept with animal pathogens is promising
- transgenic mosquitoes blocking a human plasmodium not developed
- no reports of plant pathogens targeted
    - potential hosts such as glassy-winged sharpshooter and Asian citrus psyllid have not been transformed
- vector population replacement
    - *Medea* 'selfish gene' system only tested in Drosophila

Status, challenges and future directions for use of transgenesis to control arthropods and their pathogens

Challenges and Future directions

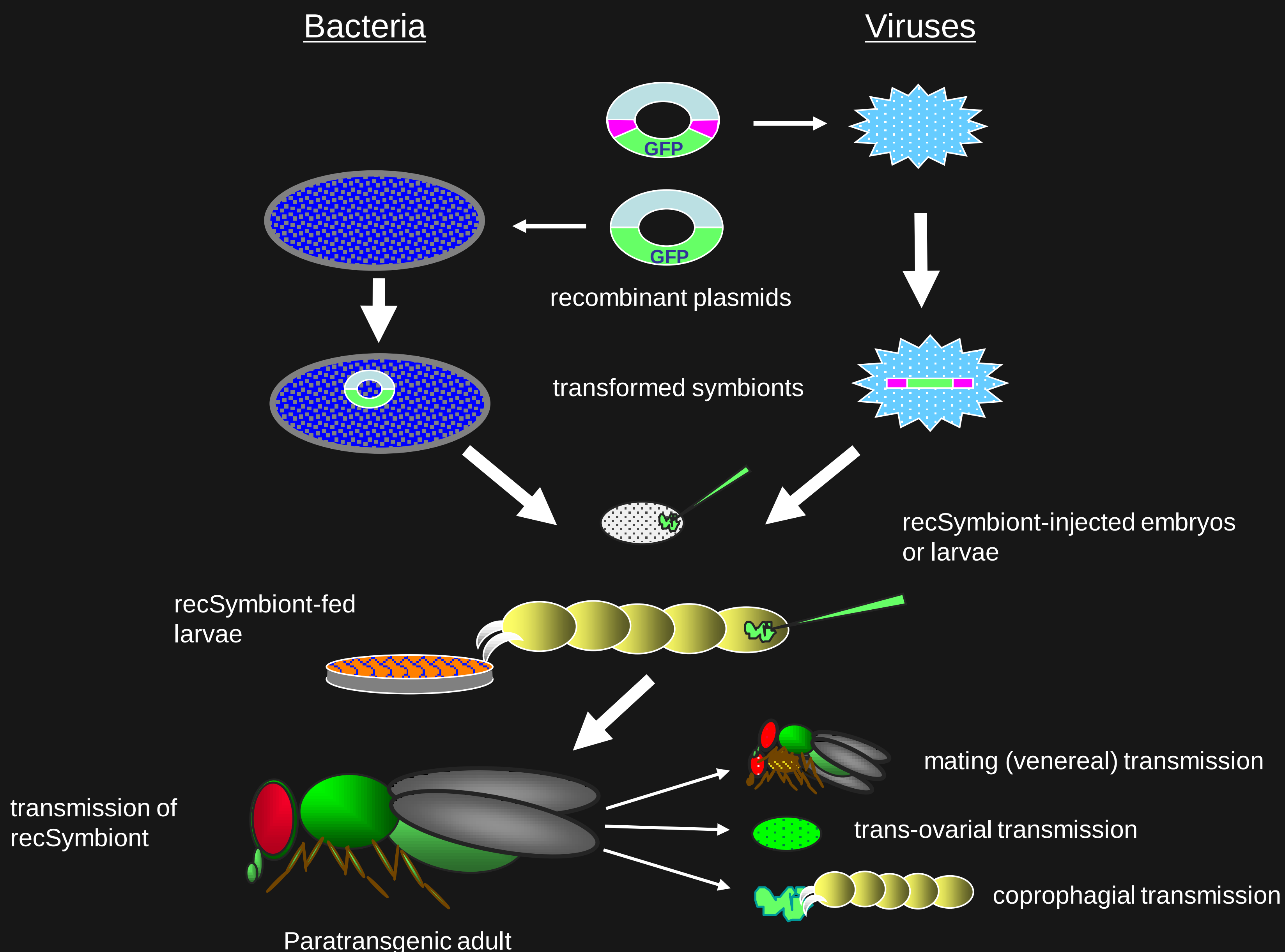
- not all pest insects amenable to transgenic methodology
  - new DNA delivery methods to expand species amenable to gene-transfer
- potential ’genetic breakdown' of transgene phenotype after mass rearing
  - test for transgene reversion/modifications; use of dual systems
- human parasite control awaits testing
  - expand testing; improve *P. falciparum/vivax* culture
- reliable 'gene drive' system not available
  - further testing of artificial *Medea* system
- risk issues for ‘spread’ transgenes not addressed
  - further large-scale testing and modeling

Paratransgenic approaches to arthropod animal/plant pathogen control

- transformation of bacterial or viral symbiont
  - to kill the pathogen
  - to inhibit vectorial capacity of host

- types of control
  - bacterial/viral - expression of antibodies to disease vectors
  - viral-mediated RNAi to pathogen development
  - bacterial/yeast expression of lytic peptides to host or pathogens
  - yeast expression of anti-viral molecules

Paratransgenesis: transformed symbionts



Paratransgenic approaches to arthropod control

Host species	Symbiont	Transgene	Target	Pathogen
Tested				
Rhodnius prolixus	*Rhodococcus rhodnii* (bacteria)	*Cecropin A*	hindgut	*Trypanosome cruzi*
An. stephensi	*E. coli*	PLA2; [SM1]	midgut	*P. berghei*
Formosan subterranean termite	*Kluyveromyces lactis* (yeast)	lytic peptides hecate/ melittin	gut	protozoa

Paratransgenic approaches to arthropod pathogen control

Host species	Symbiont	Transgene	Target	Pathogen
Proof of Concept				
Glossina morsitans (tstetse)	*Sodalis* (bacteria)	EGFP	midgut, hemolymph	African trypanosome
An. stephensi	*Asaia sp.* (bacteria)	EGFP/DsRed	midgut, salivary gland	*Plasmodium*
Ae. aegypti	*Sindbis* virus	EGFP	fat body, muscles, nerves	(host-specific)
An. gambiae	*AgDensovirus*	EGFP	midgut, fat body and ovaries	*Plasmodium*
An. gambiae	*Sindbis* virus	EGFP	fat body, muscles, nerves	*Plasmodium*
Homalodisca coagulata (GWSS)	*Alcaligenes xylosoxidans denitrificans* (yeast)	DsRed	foregut	*Xylella fastidiosa* (Pierce's disease)
Perkinsiella saccharicida (planthopper)	*Candida*-YLS (yeast like symbiont)	EGFP/ antibiotic-resistance	fat body	Fiji disease virus (FDV)

Status, challenges and future directions for use of paratransgenesis to control animal and plant pathogens

Status

- paratransgenic systems should not be subject to transgenic insertional mutations (host fitness deficiencies), positions effects, and potential for horizontal transfer
- three paratransgenic systems using bacterial and yeast symbionts created and tested in-lab for pathogen disruption
- seven or more systems using bacteria, viral, and yeast symbionts tested for proof of concept showing symbiont transformation and vertical transmission
- potential use for RNAi strategies

Status, challenges and future directions for use of paratransgenesis to control animal and plant pathogens

Challenges

- need to put tested systems into field application (termite may be close)
- need to follow-through on successful proof-of-concept strategies
- efficient spread of symbiont throughout host population
- assuring symbiont host-specificity

Status, challenges and future directions for use of paratransgenesis to control animal and plant pathogens

Future Directions

- more follow-through on tested systems
- large-scale testing and assessment of symbiont replacement
  in natural host populations

- explore use of *Wolbachia*-induced cytoplasmic incompatibility (CI) to induce sterility
  - not paratransgenic
  - infected males induce sterility in non-infected females
  - release males infected with *Wolbachia* strain not present in field population
    - sexing is essential (unlike SIT; may require transgenic sexing strains)
    - infect insect vectors with *Wolbachia* that reduce lifespan (eg Dm-wMelPop) decreasing pathogen's ability to mature
  - use in paratransgenesis exciting prospect
    - will require artificial culture and transformation

Prospects and Possibilities for the Use of Transgenesis and Paratransgenesis

- a lot of exciting work on model systems and proof-of-principle
- number of strains and nearness to application further advanced for transgenic approaches – but only a few in field release
- paratransgenesis provides useful opportunities that may be of less social, ecological and regulatory concern
- more emphasis on risk analysis for both approaches